# SEPARATION AND QUALITATIVE RECOVERY OF MAJOR PROTEINS FROM A SINGLE SAMPLE OF SKELETAL MUSCLE

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## ABSTRACT

The major soluble and myofibrillar proteins of skeletal muscle were separated into five fractions by extracting a single sample with solutions of increasing ionic strength and pH. After separation of myoglobin, other soluble proteins, and myosin, an acetone powder was prepared from the residue; the extractions were continued to yield actin and the troponin-tropomyosin complex. From 200 g of skeletal muscle the average recoveries were: total sarcoplasmic proteins, 4.5 g; myoglobin, 0.55 g; myosin, 2.7 g; actin, 0.1 g; and troponin-tropomyosin complex, 17.5 mg. The method was designed for investigating the effects of physical or chemical treatment of whole muscle or whole animals by monitoring changes in individual muscle proteins. This is particularly desirable for comparisons of amino acid composition, since naturally occurring levels of methylated histidine and lysine vary in vertebrate muscle among species, among individual members of a species, and among muscle types.

## INTRODUCTION

Skeletal muscle exhibits a highly specialized ultrastructure; each muscle cell contains complex bundles of myofibrillar proteins, myoglobin, $Ca^{2+}$-ATPase for transporting $Ca^{2+}$ into the sarcoplasmic reticulum, and a variety of enzymes for supporting metabolic activity. Many procedures for obtaining these proteins have been reported, but few are designed for recovering all major skeletal muscle proteins from the same sample. Proteins obtained by solubilizing myofibrils with detergents[2,3] are often not suitable for further analysis and study because of difficulties associated with removing detergents after preparative steps. Other methods[4,5] employ reagents which could have deleterious effects on reactive amino acid residues.

For studies of possible chemical modifications induced in skeletal muscle proteins by treating whole muscle with chemical reagents, it was necessary to obtain fractions containing myoglobin and major myofibrillar proteins from the same sample. The procedure described here takes advantage of solubility differences among the cytoplasmic and myofibrillar components of muscle[6] by employing sequential extractions with solutions of increasing ionic strength and pH, followed in each case by appropriate separation techniques. The final recoveries are made from an acetone powder prepared after removal of myoglobin, cytoplasmic enzymes, and myosin. The success of the method results from the choice of conditions for each fractionation step which do not adversely affect subsequent procedures.

## MATERIALS AND METHODS

Bovine semitendinosus muscle, from animals killed 15 to 24 h previously and refrigerated, was obtained at a nearby abatoir and kept iced throughout initial preparations. Other operations, except as noted, were carried out at 5° C. All reagents used were of the highest quality obtainable. ATP (sodium salt), dithiothreitol (DTT)[7], and Aquacide were products of CalBiochem/Behring[8]; phenylmethylsulfonyl fluoride (PMSF), obtained from Sigma, was dissolved in isobutyl alcohol at a concentration of 0.1 M and kept frozen until just before use. Deionized water was further treated on additional deionizing and charcoal columns followed by distillation.

Water free of $CO_2$ was prepared by boiling distilled deionized (DD) water for 30 min. A drying tube containing 6- to 8-mesh Ascarite (Fisher Scientific) was inserted into the neck of the flask which was then cooled rapidly. A layer of glass wool and indicating Molecular Sieve (Molindicator, Coast Engineering Laboratories, Gardena, CA) at the inner end of the drying tube prevented contact of water vapor with the Ascarite. The pH of $CO_2$-free water was always 7.00.

To minimize contamination by microorganisms, extraction solutions were filtered through 0.22-micron Millipore filters. Solutions of recovered proteins were concentrated, when necessary, using either $N_2$-driven Amicon stirred concentrators with Diaflo membranes, Aquacide treatment of protein solutions contained in dialysis membranes, or a collodion bag vacuum concentrating apparatus (Schleicher and Schuell). Measurements of pH were made with a Radiometer Model 82 pH meter, and a Perkin-Elmer Model 559 spectrophotometer was used for ultraviolet-visible spectra. Centrifugations were conducted in a Beckman Model L8-70 ultracentrifuge or a Sorvall Model RC2-B high-speed centrifuge, as appropriate.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was conducted in 125-mm tubes (7 mm I.D.) using stacking gels, as in the Laemmli procedure[9]. Two formulations for separating gels were designed to accommodate the wide range of molecular weights in muscle protein subunits (17,000 to 200,000)[10]. For high molecular weights, the gels contained 10% acrylamide monomer and were prepared with 0.75 M tris buffer pH 8.8, whereas for low molecular weights (10% acrylamide monomer), 1.5 M tris buffer was used. In both cases, 20% of the acrylamide content of the stacking gels was N,N'-methylenebisacrylamide cross-linker.

Protein standards were purchased from Boehringer/Mannheim, Miles Laboratories, Sigma, or Worthington. To enhance the detection of minor components protein bands were stained with Coomassie brilliant blue R250 containing 1.0% copper chloride[11]. The molecular weights of proteins in the bands were obtained from linear relationships between log molecular weight and mobility[12] established with standard proteins. Polyacrylamide gel electrophoresis was also conducted in the absence of SDS[13] to test for the presence of hemoglobin in the myoglobin recovered.

Assay for Intact Mitochondria. To detect conditions which might rupture mitochondria, supernatant solutions were assayed for citrate synthetase[14] before and after freezing and thawing.

## EXTRACTION PROCEDURES

The fractionation scheme organizes established methods for preparing myofibrillar proteins into a sequential procedure (Figure 1); buffers and solutions used throughout the separation steps are listed in Table I. An ultraviolet-visible spectrum was obtained for each protein fraction after recovery and the concentration estimated using the appropriate extinction coefficient

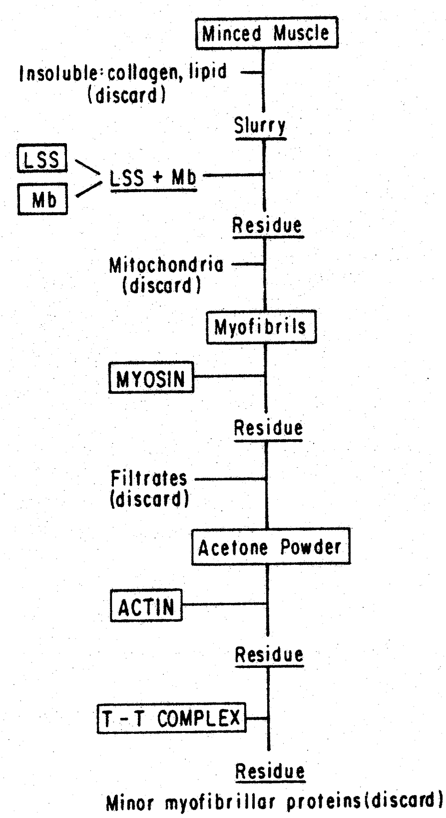



FIGURE 1

Schematic diagram of procedures for extracting skeletal muscle.

TABLE I

Extraction Solutions

| Solution | Composition | pH |
|---|---|---|
| A | 0.1 M KCl + 0.01 M $KH_2PO_4$ | 5.6 |
| B | 0.1 M KCl + 0.01 M $KH_2PO_4$ | 6.0 |
| C | 0.1 M KCl | |
| D | 0.6 M KCl + 0.20 M $KH_2PO_4$ | 6.6 |
| E | 0.048 M $NaHCO_3$ + 0.1 mM $CaCl_2$[a] | |
| F | 0.01 M $NaHCO_3$ + 0.01 M $Na_2CO_3$ + 0.1 mM $CaCl_2$[a] | |
| G | $CO_2$-free water[b] | 7.0 |
| H | 0.01 M Na phosphate + 0.04 M NaCl | 7.0 |
| J | 0.1 M tris + 0.2 mM $CaCl_2$ + 0.5 mM ATP + 0.2 mM DTT | 8.5 |
| K | 0.003 M $NaN_3$ + 0.25 mM ATP[a] | |
| L | 0.01 M tris + 0.25 mM ATP; add PMSF to 0.1 mM[c] | 8.0 |
| M | 0.01 M tris + 0.25 mM ATP + 0.5 mM DTT + 0.006 M $NaN_3$ | 8.0 |

[a]pH not critical. [b]See text for details of preparation. [c]PMSF solution described in text.

from Table II. Spectra with high baselines were first subjected to a computerized version[15] of the algorithm of Leach and Scheraga[16] to eliminate the contribution of scattering.

Low Salt Soluble Fraction. After connective tissue and excess fat were dissected away, the muscle was minced coarsely in a sterilized meat grinder; 200 g of minced muscle was divided among four 500-ml Erlenmeyer flasks, 200 ml of Buffer A was added to each, and the resulting slurries were stirred overnight (14 to 16 h) to extract Mb and sarcoplasmic proteins[6]. The combined slurries were

TABLE II

Extinction Coefficients for Determining Protein Concentrations

| Protein | Wavelength, nm | $\varepsilon, M^{-1}$ | Reference |
|---|---|---|---|
| $MbO_2$ | 580[a] | $1.44 \times 10^4$ | 32 |
| | 542[a] | $1.39 \times 10^4$ | 32 |
| | 415[b] | $1.28 \times 10^5$ | 32 |
| metMb | 630[a] | $3.9 \times 10^3$ | 32 |
| | 502[a] | $1.02 \times 10^4$ | 32 |
| | 408[a] | $1.88 \times 10^5$ | 32 |
| Myosin | 279 | 5.55[c] | 33 |
| Actin | 280 | 11.49[c] | 34 |
| T-T complex | 278 | 3.80[c] | 29 |

[a]Horse skeletal muscle. [b]Sperm whale skeletal muscle. [c]$E^{1\%}$.

then filtered through fine cheesecloth to remove insoluble collagen and lipid, and the filtrate was centrifuged for 1 h at 90,000 X g. The supernatant was filtered by gravity through a glass fiber filter to remove traces of residual lipid and then dialyzed against Buffer B for 48 h with four changes of buffer to remove components of molecular weight lower than 12,000, the cutoff limit of the dialysis tubing. Treatment of the myofibril pellets is discussed below.

Myoglobin was separated from sarcoplasmic proteins in the dialyzed supernatant solution by gel filtration on a column of Bio Gel A .5 (Bio Rad); the column was eluted with Buffer B.

Myofibrils. The combined pellets from the first centrifugation were homogenized with 10 times their volume of Solution C for 3 min at high speed in a blender to release mitochondria from the myofibril mass. Centrifugation for 1 h at 8000 X g followed, and the supernatant containing mitochondria was discarded.

Minimizing the time for extracting myosin from the myofibrils with high ionic strength buffer leaves most of the actin intact in the myofibrils[6]. Pelleted myofibrils were slurried with a 10-fold volume of Buffer D by a few 3-sec high-speed pulses in the blender. Centrifugation for 30 min at 90,000 X g produced compact pellets with crude myosin as the supernatant.

Acetone Powder. Preparation of acetone powders of animal tissues is an established technique which enhances recovery of some proteins in an undenatured form[17]. The method described here is an adaptation of Straub's procedure[18]. The pellets remaining after extraction and separation of myosin were suspended in five times their volume of Buffer E, stirred for 30 min, and filtered by gravity through cheesecloth. The filtrate was discarded, as were all other filtrates produced during the preparation of acetone powder. The residue from the first filtration was resuspended in an equal volume of Buffer F and stirred for 10 min, after which the solution was diluted (with constant stirring) by the addition of 10 volumes of DD water; this solution was again filtered by gravity through cheesecloth. Two volumes of 0° C acetone (Mallinckrodt Nanograde) were added to the residue with stirring for 10 min, followed by filtration through cheesecloth. The acetone extraction was repeated five more times; the final residue was dried briefly in the hood and then overnight in a vacuum desiccator. The dried powder was stored frozen at -30° C.

Extraction of Acetone Powder. Low temperature extraction of actin from acetone powder prevents contamination by the troponin-tropomyosin (T-T) complex[19] To 4 g of acetone powder in a beaker maintained at 0° C, 100 ml of cold $CO_2$-free water (G, Table I) was added with stirring for 10 min. The solution was filtered by suction through cheesecloth on a Buchner funnel or centrifuged at 23,500 X g; a combination of both procedures was used if the amount of residue was large. The residue or pellets were washed with an additional 50 ml of G and filtered again. The residue contained the T-T complex, and the procedure for its further treatment is described below.

The supernatant, containing G-actin, was centrifuged for

0.5 h at 105,000 X g to clarify and then concentrated by placing it in dialysis tubing and surrounding the dialysis bag with Aquacide powder in a pan. Polymerization of G-actin to F-actin was facilitated by this preliminary concentration step. After the volume of G-actin was reduced by about one-third, it was transferred to a glass-stoppered graduated cylinder and its volume measured. Solid KCl and $MgCl_2$ were added, as in the procedure of Spudich and Watt[20], to concentrations of 0.05 M and 0.002 M, respectively. The solution was allowed to stand 2 h at room temperature, after which the KCl concentration was increased to 0.6 M and the solution stirred gently for 1.5-2 h at room temperature. The polymerization reaction is favored by increasing the ionic strength to screen the electrical charges on G-actin and by the presence of $Mg^{2+}$, which may induce a specific conformation of G-actin more favorable for polymerization[21].

After the onset of cloudiness, indicating F-actin formation, the solution was centrifuged for 3 h at 80,000 X g. The pelleted F-actin was then resuspended in a small amount of Buffer H by gentle homogenization in a Potter-Elvehjem tissue grinder; the resulting suspension was either depolymerized to G-actin by dialysis against Buffer J (which lowers the ionic strength and depletes $Mg^{2+}$) or stored in the polymerized form with Buffer K.

The wet residue from the extraction of the acetone powder, containing the T-T complex, was placed in a beaker and treated by the addition, with stirring, of 150 ml of Buffer L, followed immediately by the addition, with vigorous stirring, of PMSF to a final concentration of 0.1 mM. The beaker was covered and gentle stirring resumed at room temperature for 3 h to extract T-T complex. After filtration by suction through cheesecloth on a Buchner funnel and an additional wash of the residue by 75 ml of Buffer L, the slightly colored solution was clarified by centrifuging for 10 min at 23,500 X g. The residue, containing minor myofibrillar proteins, was discarded.

The supernatant was transferred to a 600-ml beaker and set in an ice bath with the pH electrode immersed so that pH could be maintained at 7.00. After the solution was brought to pH 7.00 with concentrated HCl or $NH_4OH$, solid $(NH_4)_2SO_4$ was added with gentle stirring to a concentration of 40% (w/v) to salt out any remaining traces of actin; the precipitate was removed by centrifugation at 23,500 X g for 10 min. The supernatant, again in an ice bath and with pH maintained at 7.0, was treated with additional solid $(NH_4)_2SO_4$ to 65% (w/v) by gently stirring. The final pellet containing the T-T complex was isolated by centrifuging at 23,500 X g for 10 min. The pellet was resuspended in Buffer M and dialyzed against the same buffer. Material which precipitated out during the dialysis was removed by centrifugation. The solution of T-T complex was stored at 5° C.

## RESULTS

Yields of myoglobin, cytoplasmic proteins, and myosin were generally ample (Table III), but yields of actin and T-T complex depended on successful preparation of the acetone powder, a critical step. When properly prepared the powder was composed of tiny white particles; less successful preparations containing large grey particles produced only small amounts of actin and T-T complex.

Immediately after dialysis, the fraction containing low salt soluble proteins plus Mb (LSS + Mb) had a visible spectrum which indicated that the heme protein present was mainly oxymyoglobin ($MbO_2$). SDS gel electrophoresis of LSS + Mb produced 14 distinct bands plus additional faint bands (Figure 2, gel I). Proteins in these bands ranged in molecular weight from about 17,000 to 100,000 (Figure 3) and were assumed to include soluble enzymes of glycolysis, creatine kinase, Mb, and traces of serum proteins[22]. The first peak to emerge during elution of LSS + Mb from Bio Gel A (LSS), had an SDS electrophoresis pattern showing one band absent (Figure 2, gel III). The second peak from Bio Gel A was reddish and the visible spectrum indicated it to be mainly metmyoglobin (metMb). It produced a single band in SDS electrophoresis (Figure 2, gel II) which had a mobility similar to that of the band absent in gel III, Figure 2, and which corresponded to a molecular weight of about 17,000 (Figure 3, Curve b).

From 200 g of skeletal muscle, an average of 4.5 g of sarcoplasmic proteins (LSS) and 0.55 g of Mb were recovered (Table III); the Mb value was based on the absorbance of the Soret band in the visible spectrum of the second peak from Bio Gel A. Gel electrophoresis in the absence of SDS showed that the Mb recovered contained a trace of hemoglobin when compared with a bovine hemoglobin standard.

PROTEINS FROM A SAMPLE OF SKELETAL MUSCLE

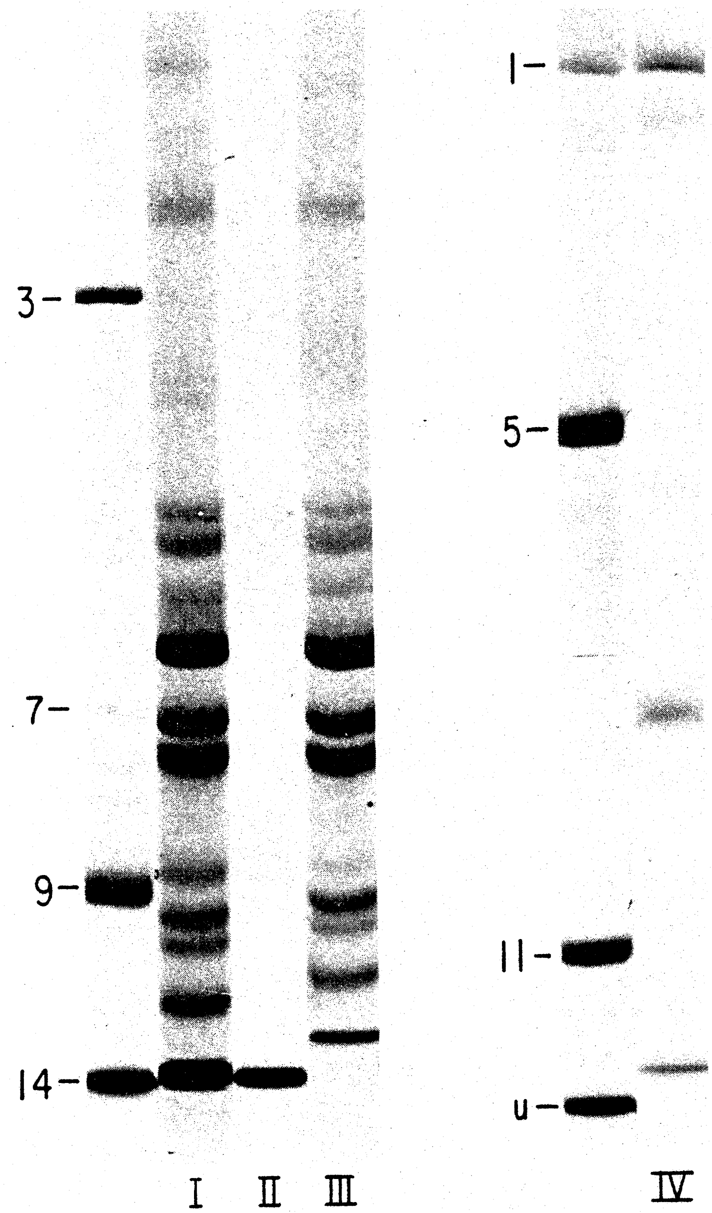
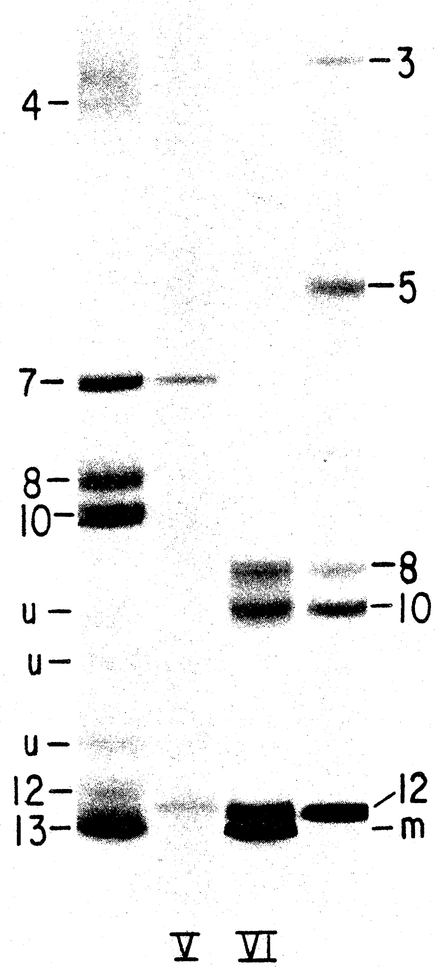



FIGURE 2



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The solution containing crude myosin was typically cloudy and opalescent and had a substantial scattering baseline in the ultraviolet spectrum which was corrected as described. Based on the absorbance of the corrected spectrum at 278 nm, the recovery of myosin averaged 2.7 g from 200 g of muscle (Table III). SDS gel electrophoresis (Figure 3, Curve a) showed a slight contamination by actin and occasionally traces of protein in minor bands (as in gel IV, Figure 2); the lower three bands in gel IV, Figure 2, may be myosin light chains[23].

Conditions for the polymerization and centrifugation of F-actin were carefully controlled to prevent the formation of a rubbery pellet which could be depolymerized only with great difficulty. Based on the ultraviolet spectrum of G-actin after depolymerization, 0.1 g of actin was usually isolated from 4 g of acetone powder (Table III). SDS gel electrophoresis (Figure 2,

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FIGURE 2

SDS polyacrylamide gel electrophoresis of molecular weight standards and protein fractions separated from bovine skeletal muscle. To improve the resolution of lower molecular weight proteins, 10% gels were prepared with 1.5 M tris buffer (see text for details).

I. LSS + Mb fraction. II. Mb after elution from Bio Gel A. III. LSS after elution from Bio Gel A. IV. Myosin. V. Actin. VI. T-T complex. u. Unknown contaminant. m. Troponin C not observed in standard.
Standards and molecular weights: 1. myosin heavy chain (200,000); 2. β-galactosidase (130,000); 3. phosphorylase b (92,500); 4. glucose oxidase (80,000); 5. bovine serum albumin (68,000); 6. pyruvate kinase (57,000); 7. actin (42,000); 8, 12, 13. troponin, subunits T (37,000), I (22,000), and C (18,000); 9. lactate dehydrogenase (35,000); 10. tropomyosin (35,000); 11. carbonic anhydrase (31,000); 14. myoglobin (17,200).

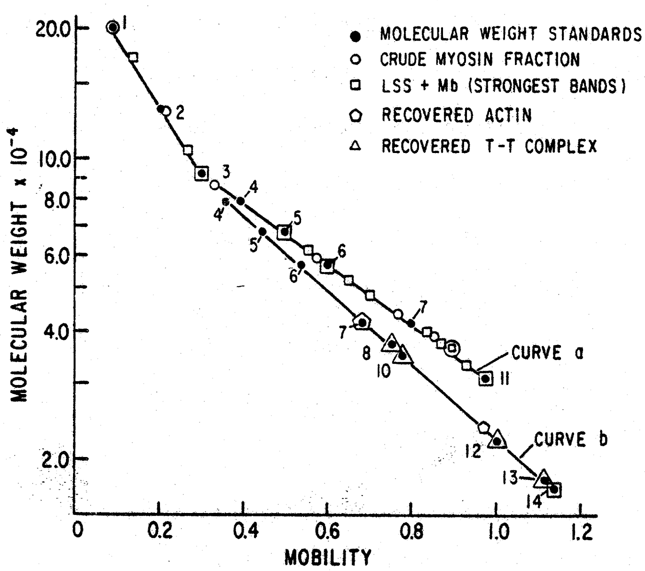


FIGURE 3

Determination of molecular weights of proteins recovered from bovine skeletal muscle.
Curve a. Using gel formula for resolution of high molecular weight proteins.
Curve b. Using gel formula for resolution of low molecular weight proteins. Standards numbered as in Figure 2.

gel V; Figure 3, Curve b) showed that only minor impurities were present.

Recoveries of T-T complex were relatively smaller than those of Mb, LSS proteins, myosin, and actin; further refinement of techniques should lead to improved recoveries. However, only 12% of skeletal muscle myofibrils is T-T complex[23]. The ultraviolet spectrum of the complex was used to calculate its concentration; approximately 17.5 mg of T-T complex was obtained from 4 g of acetone powder. The three subunits of troponin plus tropomyosin

TABLE III

Recoveries of Bovine Skeletal Muscle Proteins

| Protein | Source | Skeletal Muscle Protein, g | | |
|---|---|---|---|---|
| | | Recovered[a] | Theoret.[a] | Reference |
| Mb | Buffer A extract | 0.55[b] | 1.4[c] | 35 |
| LSS | Bio Gel A elution of Buffer B dialysate | 4.5[d] | 47[e] | 22 |
| Myosin | Solution C extract | 2.7 | 13[e] | 22 |
| Actin | Solution G extract of acetone powder | 0.1[f] | 5.2[g] | 36 |
| T-T | Buffer L extraction of residue after actin recovery | 17.5 mg[f] | 2.8[g] | 36 |

[a]Average value from 200 g of minced skeletal muscle.
[b]Based on visible spectrum of Mb fraction eluted from Bio Gel A.
[c]Bovine.
[d]Estimated from weight of lyophilized aliquot of LSS + Mb (corrected for salt content) minus weight of Mb calculated from visible spectrum.
[e]Rabbit or pig. [f]From 4 g of acetone powder. [g]Rabbit.

were observed with SDS gel electrosphoresis (Figure 2, gel VI); Figure 3, Curve b).

The preparative procedure was designed to recover muscle proteins without contamination by mitochondrial enzymes, so citrate synthetase activity was used as a marker for intact mitochondria. Results of assays showed that ruptured mitochondria were not present in protein fractions recovered before acetone treatment, and the number of intact mitochondria diminished greatly after the wash with Solution C. Actin from the acetone powder had $4 \times 10^{-3}$ units of citrate synthetase activity per ml. T-T complex had none.

## DISCUSSION

The procedure described here was developed with skeletal muscle from bovine tissue partly because it was readily available but also because amino acid composition, protein sequences, and spectrophotometric constants have not been reported as frequently for this species; the use of bovine muscle would facilitate establishment of such data. All the procedures incorporated into the method described were originally devised for skeletal muscle of rabbit or rat, so applicability to skeletal muscle of other vertebrates is expected.

No difficulties should arise in scaling down the procedures for animals as small as the rat, although we have not tested the procedure with smaller animals. Recoveries of proteins from a single type of mouse skeletal muscle may be too low to be practical; however, an alternative would be establishment of average values for amino acid composition of whole mouse skeletal muscle to be used as controls.

Recoveries are not quantitative but the procedure does provide the major proteins in amounts sufficient for potential final purification. Established techniques, usually depending on ion-exchange chromatography or gel filtration, have been reported for isolation of proteins from the major fractions. Hapner et al.[24] have reported a simplified procedure for purification of myoglobin, although the fraction from Bio Gel A obtained as described here is relatively pure. Myosin can be isolated in very pure form by the procedures of Mozersky[25], Richards[26], or Chantler[27], as examples. Spudich and Watt[20] and Loscalzo et al.[28] have described procedures for purifying actin, as have Eisenberg and Kielly[29] and Cummins and Perry[30] for troponin and tropomyosin. Both actin and T-T complex recovered by the described procedure had few contaminants (Figure 2, gels V and VI).

The advantages of the isolation procedure described are many. As indicated, it is desirable to obtain all muscle proteins from the same sample for investigations in which specific physical or chemical conditions are to be imposed on whole muscle or whole animals, as in chemical modifications or exposure to environmental factors, or for studies which compare amino acid composition or levels of naturally occurring amino acid modifications—e.g., methylation. Methylation of histidine and lysine is variable in muscle proteins depending on the species, the age of the animal, and the particular muscle[31].

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7. Abbreviations used: DD, distilled and deionized; DTT, dithiothreitol; LSS, low salt-soluble proteins of sarcoplasm; LSS + Mb, LSS including myoglobin; Mb, myoglobin; $MbO_2$, oxymyoglobin; metMb, myoglobin with $Fe^{3+}$; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; T-T troponin-tropomyosin complex comprised of tropomyosin, troponin T, troponin I, and troponin C.

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